REMARKS

Claims 19-29 are pending in the application. Claims 19 and 22-25 have been amended. All claims are supported by the specification and no new matter is believed to have been added. Reconsideration of claims 19-29 is respectfully requested.

§102 and 103 Rejections.

Claims 19-20 stand rejected under 35 USC 102(b) as being anticipated by Zuagg et al and claims 21-22, 25-26 and 28-29 are rejected under 35 USC 103 (a) as being obvious over the same reference (Zaugg et al, J. Biol.Chem.1977, Vol. 252, No. 23, pages 8542-8548). These rejections are respectfully traversed.

In reciting the reference, the Examiner notes that Zaugg et al discloses a number of "aromatic aldehydes, e.g. furfural (compound 25), useful for inhibiting hemoglobin sickling" (see page 3 of Office action). And, the Examiner further notes that "the aldehyde substituent forms Schiff base (imine) linkage with amino group of intracellular hemoglobins. Hence, the antisickling effect." (see page 4 of the Office action. Since the compounds claimed in the present invention for treating sickle cell disease have an aldehyde substituent, the Examiner then summarily concludes that the claims are anticipated and *prima facie* obvious over the Zuagg et al reference.

If what the Examiner has noted above were true and correct, then any aromatic aldehyde that forms a Schiff base should have antisickling properties. However, a careful review of Zuagg et, particularly Table 1 at page 8543, clearly reveals that not all aromatic aldehydes modify hemoglobin nor inhibit sickling. It is important to note in this respect that there are a number of aromatic aldehydes (see table below derived from Zuagg et al's Table 1 at page 8543) that modify

hemoglobin between 0-60 % by Schiff base formation but **do not** have much or any effect on inhibition of hemoglobin sickling (see, e.g., compound 12, 4-dimethylaminobenzaldehyde, which has no effect at all).

Compound Number	Compound name	%Hb Modified	% Δp50 mmHg Change
1	Benzaldehyde	60	5
8	2- Carboxybenzaldehyde	15	5
9	4- Carboxybenzaldehyde	20	0
12	4-Dimethylaminobenzaldehyde	0	0
17	5-Nitrosalicylaldehyde	0	55
24	Pyridoxal Phosphate	0	0
31	2- Hydroxyacetophenone (a ketone)	0	0

Note: The Compound Number in the above Table corresponds to the compound number in Zuagg et al's Table 1 at page 8543.

Thus, it ought to be clear from the above that Zuagg et al itself **teaches away** from the conclusion that **simply because a compound has an aromatic aldehyde substituent and forms Schiff base linkage, hence it will be efficacious in treating sickle cell disease.** Indeed, an aromatic aldehyde hemoglobin modifier is of no use for sickle cell anemia or hypoxia, even though it forms a Schiff base, unless it affects p50 of hemoglobin. Even more contrasting is the fact that one of the aldehydes included in Zuagg et al study (compound number 17, viz., 5-nitrosalicylaldehyde) does not even modify hemoglobin (by forming a Schiff base) but inhibits sickling. In other words, contrary to the Examiner's assertion, it cannot be *a priori* assumed that all aromatic aldehyde compounds are useful for inhibition of hemoglobin sickling. For the above reason alone, through its self-revealing study, Zuagg et al reference teaches away from the inference made by the Examiner.

More importantly, applicant has presented experimental data in several Tables (see, e.g., Table 2 on page 23, Table 3 on page 24 and Table 4 on page 25 of the specification) comparing antisickling efficacy of various compounds that are subject of the instant claims. These include furfural (abbreviated as FUF); 5-Methylfurfural (abbreviated as 5MF), 5-Ethylfurfual (abbreviated as 5EF); and 5-hydroxymethyl-2-furfural (abbreviated as 5HMF), showing unexpected superior

antisickling properties of these compounds relative to furfural.

For instance, the data for oxygen equilibrium studies of these compounds with normal human whole blood (AA) cells (Table 2, column 3, and discussion on page 23 of the specification) clearly indicates that 5HMF is a superior antisickling agent than furfural (FUF). At 5 mM concentration, the Δp50 value of 5HMF is -17.52 mmHg while that of FUF is -11.35 mmHg. The other alkyl derivatives such as 5MF and 5EF are also more potent and better antisickling agents than furfural as they have higher Δp50 values than that of furfural. P50 is the measure of 50% oxygen saturation in the presence or absence of a compound and the higher (negative) the p50 shift, the better is the ability of a compound to left shift the oxygen dissociation curve (ODC) and therefore greater is the antisickling capability.

The oxygen equilibrium studies data with homozygous sickle red blood (SS) cells (Table 3, columns 3 and 5, and discussion on pages 23-24 and Figure 3) again clearly exhibits that 5HMF is allosterically most potent antisickling agent (Δp50 = -25.2 mmHg) than FUF (Δp50 = -15.8 mmHg) at 5mM concentration. Figure 3 in the specification shows that ODC curve of 5HMF is significantly left shifted compared with the ODC of FUF.

5HMF and FUF were also evaluated for their ability to shift the ODC of Hb SS cells hemolysates. The data suggest that these compounds follow the same trend as observed during the normal whole blood studies (see Table 3, column 5, and discussion on page 24). 5HMF causes the largest Hb left shift (Δp50 = -9.4 mmHg) than FUF (Δp50 = -5.9 mmHg) and therefore 5HMF is superior to FUF.

In another *in vitro* study undertaken to determine the transport mechanism of compounds through homozygous sickle red blood cells measuring the % hemoglobin modification associated with left shifting ODC ability of the compounds, it was found that 5HMF modified the hemoglobin S to the greatest

degree (70%, Table 3, column 6, and discussion on page 24) as compared to FUF (24%, Table 3, column 6, page 24). Furthermore, Figure 4 (cation exchange HPLC analysis) in the specification shows the effect of 5HMF and FUF on the hemolysate of SS cells at 5mM concentration incubation and ability to form HbS adducts.

In another significant antisickling study, upon exposure of homozygous SS cell suspensions to only 4% oxygen, in presence of 1, 2 and 5 mM of 5HMF and FUF compounds, 5HMF inhibited sickling the most (90%) and again proved to be superior to FUF. The inhibition of sickling by 5HMF seems to be concentration dependent as shown in Table 4, column 3, and discussed on page 25 of the specification. In contrast, FUF did not inhibit Hb SS cells sickling at lower concentrations such as 1 and 2 mM.

Some of these results have been published in peer reviewed national and international journals by M. Safo et al in *J. Med. Chem.*, **47**, 4665-4676 (2004) and *Br. J. Hematol.*,**128**, 552-561 (2005) and reprints of both publications are attached hereto and made a part hereof.

In summary, the results of the comparative studies noted herein above clearly show that 5HMF is a superior antisickling agent than FUF. For convenient review the supporting data from Tables 2, 3 and 4 are being consolidated and presented in Table A below.

Table A

Compound	**ΔP50 mmHg AA Cells**	**ΔP50 mmHg SS Cells**	**ΔP50 mmHg Hemolysate**	**%HbS Modified**	**%Inhibition of sickling**
Furfural (FUF)	-11.35	-15.8	-5.9	24±5.7	30±7
5-Hydroxymethyl-2-furfural (5HMF)	-17.52	-25.2	-9.4	70±10	90±5

Further *in vivo* and *in vitro* advanced studies with 5HMF confirm the finding that 5HMF is a significantly superior and potent antisickling agent. Some of these results are discussed below in support of the instant claims and are also included in the specification on pages 31-32 and figures 5-7. These include:

(A) 5HMF has high bioavailability after a single oral dose to Transgenic (Tg) Mice.

To study the bioavailability of 5HMF, three groups of Tg sickle mice (four mice per dosage group) were given single oral administrations of 5HMF (of 50, 100 or 200 mg/kg body weight). The results of HPLC analyses of plasma and RBC lysates obtained from whole blood samples of treated Tg sickle mice showed high levels of unchanged 5HMF after a single oral dose. The pharmacokinetic parameters are shown in Table B above. The mean AUC value increased proportionally with the administered dose (r2 ¼ 0.95). Upon administration of the three different doses, the apparent clearance (Cl) did not significantly differ, while the steady state volume of distribution (Vdss) gradually decreased from 652.4 to 453.1 ml/kg as the dose of 5HMF decreased. The time to achieve maximum concentration (Tmax) was 0.5 h, while the half-life (T1/2) was approximately 1.5 h in mice that received 100 or 200 mg/kg body weight; however, the T1/2 was reduced by almost half in those mice that received 50 mg/kg body weight 5HMF.

Table B. Pharmacokinetic parameters of 5HMF afte oral administration of 50, 100 or 200 mg/kg t transgenic sickle mice.

	5HMF concentration (mg/kg)		
Parameter	50 ($n = 4$)	100 ($n = 4$)	200 ($n =$
AUC (μg/ml/min)	133 ± 35·5	411·7 ± 24·6	663·5 ± 1
$T_{1/2}$ (h)	0·83 ± 0·17	1·5 ± 0·6	1·5 ± 0
C_{max} (μg/ml)	68 ± 10·9	180·7 ± 32·6	305·7 ± 2
C_{max} (mmol/l)	0·54 ± 0·09	1·43 ± 0·26	2·43 ± 0
$V_{d(ss)}$ (l/kg)	0·45 ± 0·06	0·52 ± 0·07	0·65 ± 0
MRT (h)	1·8 ± 0·36	2·3 ± 0·16	2·1 ± 0
Cl (ml/kg/min)	5·2 ± 1·15	4·2 ± 0·25	5·2 ± 0

Values are given as mean ± SD.
AUC, area under the plasma concentration curve; $T_{1/2}$, termi half-life; C_{max}, maximum plasma concentration; $V_{d(ss)}$, volume distribution at steady state; MRT, mean resident time; Cl, appar clearance.

(B) 5HMF prolonged the survival time of Tg sickle mice under hypoxic conditions

5HMF showed a potent anti-sickling effect in Tg sickle mice that were exposed to hypoxia (5% O2). Without treatment, transgenic sickle mice exposed to 5% oxygen died within 15 min because of pulmonary sequestration by sickled

cells. However, only one of the eight 5HMF-treated mice died under severe hypoxic conditions; the remaining seven mice survived through the full experimental period of 60 min (survival proportion: 0.875). Upon exposure to hypoxia, the percentage of sickled cells in the blood of the untreated mice increased sharply, followed by a sharp decrease before death, while the percentage of sickled cells in the blood of 5HMF-treated mice increased slowly to a level of approximately 25%, which was maintained for the full experimental period of 60 min in seven mice. The sickled cells found in the blood of drug-treated mice seemed to be the so-called partially oxygenated sickled cells (POSCs) with 'blunt' edges. POSCs are flexible and can pass through capillaries without causing vaso-occlusion.

(C) 5HMF has no detectable adverse effects on human RBCs

Incubation of human SS cells with as high as 5 mmol 5HMF did not cause hemolysis of RBCs, nor promote oxidation or denaturation of intracellular HbS. The nystatin loading test showed that 5HMF had no noticeable effects on the Na–K pump, Na–H exchange and Na–K–2Cl co-transport activities.

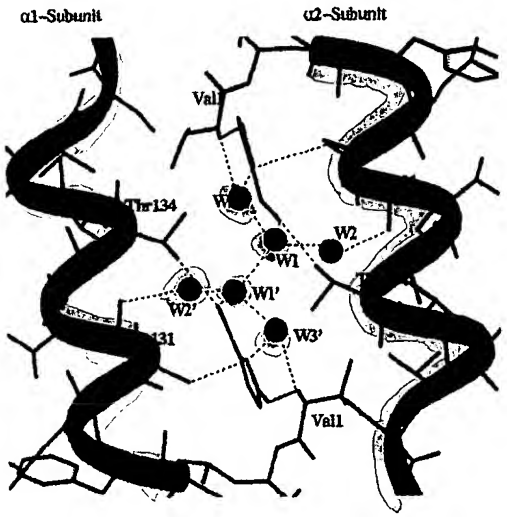
Preliminary studies, performed to determine possible inhibitory effects of plasma proteins on the binding of 5HMF with intracellular HbS, showed that the amount of modified Hb was identical in suspensions of washed RBC in either plasma or saline (final hematocrit ¼ 25% in either sample) incubated with equimolar amounts of 5HMF. The result of this preliminary experiment is indicative that plasma proteins do not inhibit the binding of 5HMF to intracellular hemoglobin.

Also significant is the fact that 5HMF does not dehydrate human SS cells. Polymerization of HbS and the sickling of SS cells are linked to the intracellular concentration of HbS. Therefore, any agent that causes dehydration of RBCs would increase the molar concentration of HbS, and presumably increase polymer formation. Furthermore, 5HMF does not promote formation of met Hb or membrane associated denatured Hb (see discussion on page 28).

(D) X-ray Crystallographic Results: A comparison of X- ray crystal structures of FUF and 5HMF-Hemoglobin complexes is discussed below and on pages 19-22 in the specification. The X-ray crystallographic binding results provide the answer as why 5HMF is more superior and specific antisickling agent than furfural or any other aromatic aldehydes that have been studied. 5HMF binds covalently to the N-terminal Val1α of the hemoglobin as envisioned, but there is raft of water molecules that provide a strong hydrogen bonding network to secure the potential drug in its active site. The mode and differences in the mechanism of action of FUF and 5HMF is presented below.

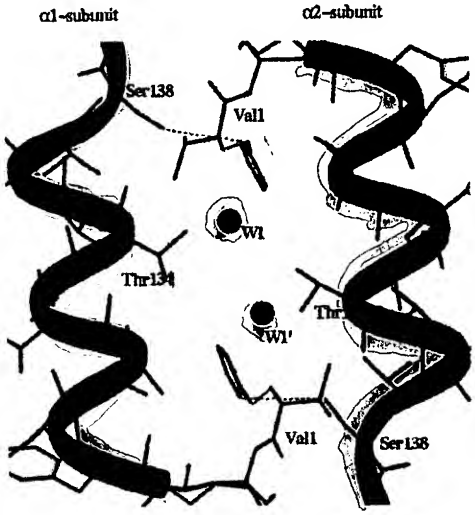
(E) Mode of Action of 5HMF and Furfural and hemoglobin binding differences: Even though 5HMF and furfural like most aldehyde antisickling compounds bind at the N-terminus of Hb at the α-cavity, the mode of interactions to hemoglobin is different resulting in the significant differences in their antisickling properties (Safo, et al., 2004, *J. Med. Chem.*, **47**, 4665-4676).

Fig. 1



5HMF

Fig. 2



Furfural (FUF)

Shown above is the crystallographic binding site of 5HMF and furfural with oxygenated Hb. Figure 1 is the binding of 5HMF (colored yellow) between the

two α-subunits of Hb. Figure 2 is the binding of furfural (colored yellow) between the two α-subunits (colored magenta and blue). As the above figures show, 5HMF and FUF bind in a symmetry related fashion at the α cleft of the two N-terminal αVal1 residues. The strong network of hydrogen bonding between six water molecules and 5HMF explains why 5HMF is biologically more potent antisickling agent. This kind of hydrogen bonding is absent in furfural, leading to less antisickling activity.

The mechanism of antisickling effect of furfural and 5HMF depends on the mode of binding to deoxy Hb (or T-state) and oxygenated Hb (or Relaxed state). The two compounds bind to deoxy Hb and destabilize it resulting in increased amount of high affinity Hb (see above). High affinity Hb, unlike low-affinity Hb does not sickle. Both compounds also bind to oxygenated Hb with the aldehyde forming Schiff-base interaction with the N-terminus nitrogen. However, while 5HMF binding stabilizes the relaxed Hb and consequently increases the high affinity Hb, binding of furfural to the oxygenated Hb on the other hand does not lead to any significant stabilization of the oxygenated Hb. Because of the 5-hydroxyl substituent on 5HMF, this compound is able to make a *novel* network of water-mediated (water colored red) hydrogen-bond interactions that stabilize 5HMF binding as well as tie the two α-subunits of the oxygenated Hb together.

It should be noted that furfural lacks the 5-hydroxyl substituent and thus unable to make these inter-subunit water-mediated interactions. In addition, the 5-hydroxymethyl substituent of 5HMF also makes a strong intrasubunit hydrogen bond interactions with α1Thr134 (2.6A°) and this interaction is absent in the FUF-Hb complex structure. As a result of the high binding affinity of 5HMF, and most importantly the ability to tie the two subunits of the oxygenated Hb together, the high affinity Hb is significantly increased by 5HMF binding compared to furfural. Consequently, 5HMF is significantly more potent antisickling than furfural.

Although furfural is mentioned in Zaugg et al reference, elaborate findings set forth above clearly establish that furfural and 5HMF are quite dissimilar and have significantly different antisickling properties and they are by no means equivalent in this regard as asserted by the Examiner in rejecting the claims under 35 USC 102 or 103 over Zaugg et al reference. The same is true for 5 MF and 5EF. These differences include their binding modes to Hb, amino acid interactions, role of water enhancing the binding strength, ability to shift the oxygen affinity of hemoglobin, hemoglobin co-operativity and concentration dependent left shift of the oxygen dissociation curve, etc.

In conclusion, even though furfural may seem to have certain structural resemblance with 5MF, 5EF and 5HMF, these compounds substantially differ in their biological action and possess a wide range of different allosteric properties. This could be attributed to their characteristic differences in molecular size, steric volume, and the nature of functional group that influence their overall binding in the hemoglobin pocket. Molecular modeling and 3-dimensional structural studies indicate that, contrary to Examiner's assertion (see page 5 top, lines 1-6 of the Office action) hydrogen and alkyl groups (or hydroxy-alkyl group) are **not equivalent** but different sterically and otherwise, and occupy different amount of molecular volume with the receptor protein. Although hydrogen and alkyl groups are hydrophobic (non-polar), hydroxy-alkyl group is hydrophilic or polar and the latter can form strong hydrogen bond interactions. Thus, small differences in the pharmacophore structure can contribute significantly to the favorable biological effect or enhance the allosteric effect, depending upon the atomic level interactions with the receptor protein. Therefore, even though 5HMF and furfural seem to have structural similarity, the surprising and unexpected result found from the side-by-side comparative studies enumerated above and presented in various Tables in the specification clearly indicate that 5HMF is at least 3-5 times more effective antisickling agent than furfural. In fact, 5HMF is the most potent antisickling agent reported to date in the literature. And this result alone, without more, confers patentability to the instant claims.

In light of the above the rejections under 35 USC 102(b) and under 35 USC 103 (a) are not supportable and these rejections should now be withdrawn.

§112 Rejections.

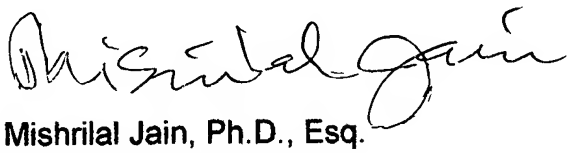
Claim 19 was further rejected under 35 USC 112, first paragraph and claims 23-25, 27 were rejected under 35 USC 112, second paragraph. The claims have been amended and it is believed the amended claims obviate these rejections.

Even though mentioned in prior submission, it is again noted that the undersigned is the **only attorney of record** and all communications related to this application be **directed to the undersigned attorney**.

The claims are now believed to be in condition for allowance and favorable action accordingly is earnestly solicited.

Should there still remain any outstanding issues; an interview with the Examiner is requested toward furthering the application for allowance.

Respectfully submitted,



Mishrilal Jain, Ph.D., Esq.
Registration No. 29315

11620 Masters Run
Ellicott City, MD. 21042-1537
Tel. 410-715-4514

January 17, 2006.